Purpose: Testing DNA for template function in the polymerase chain reaction.

Introduction: Our eventual goal is to amplify the genes/gene families (in several Glycine mx and Phaseolus varieties) in the chalcone synthase pathway. Because the varieties have not been individually sequenced there may be some optimization required in order to find primers that work (small variations in sequence at the primer binding sites will affect whether or how well the primers bind). A second challenge comes in the DNA purification process: sulfonated carbohydrates and ferric containing compounds are known to inhibit DNA polymerases. Before investing a lot of effort in optimizing primers you should make sure the DNA is an acceptable template for PCR. We make use of highly conserved genes for this purpose – sometimes called 'housekeeping' genes. Beta-actin and tubulin are frequently chosen for this purpose.

We have primers for actin and tubulin, designed against Glycine max. They did not work in our Phaseolus DNA, so a re-design is being performed. Today you will use them to determine whether your Glycine max DNA can be amplified, and next week you will test the Phaseolus DNA.

Protocol

1. Check the length and concentration of both DNAs. Since your DNA is probably about 10kbp in length, you should pour a 0.8% agarose gel in 1X TBE, and include ethidium bromide (1 ul per ml of gel solution, from a 10mg/ml stock) added when the agarose has cooled, just before pouring it into the casting tray . This will allow you to visualize the DNA as it electrophoreses, otherwise the staining can require several hours.
    a. Use the Nanodrop 2000 to determine the concentration of each DNA (do triplicates) – perform a scan and also determine whether the A260/A280 ratio indicates that you removed the RNA and protein from the sample. If you did not, perform the Phenol-chloroform extraction from the last protocol a second time.
    b. Load 2-3 ug of DNA per lane of your agarose gel (you may share a gel with a neighbor team).
    c. Use the Bionexus All-purpose Hi-Lo ladder, using 5ul per lane (it goes up to 10kbp) in the two end lanes.
    d. Run at 40V, check after 2 hours (it may require 3-4). Since the gel has a stain in it, you need only put the gel on the transilluminator and turn on the UV light.
    e. When the bands are well separated, capture an image with the digital camera and compare the band density to the markers (Bionexus includes mass as well as length). If the concentration of DNA is within 10% of the predicted mass, proceed to setting up the PCR reaction.
        i. Dilute a small amount of the DNA to a concentration of 10ng/ul with TE buffer.
2. You will test the Glycine max DNA only at this time.
    a. Actin L primer: CAT CCC CTG CCA CTA CAA CT 56.9C Tm
    b. Actin R primer: GGC CAC ACG AAG CTC ATT AT 55.4 C Tm

i. Expected product length: 450nt

c. Tubulin L primer: GGT GGC CGA GTA TCA ACA GT 57.1C Tm

d. Tubulin R primer: AAA AGG TTG GAC TGG CAA TG 53.8C Tm

i. Expected product Length: 400 nt

e. Reaction components (combine on ice, in this order). One team member will do reactions at 57C and the other at 64C. For one team member, Set up 3 tubes with the following components for Actin (using A-L and A-R primers)and 3 tubes for Tubulin (using the T-L and T-R primers). In one of each pair will you will add no genomic DNA, in order to test for primer artifacts and contamination.In one tube you will put control DNA that we know works. The final tube in the trio will contain your DNA.
Use barrier tips for pipetting. For 25ul final volume reactions add to 200ul thin-wall PCR tubes (on ice) , in this order:

i.	10X buffer	2.5 ul	
ii.	dNTP (10mM)	1.25ul	
iii.	$MgCl_2$(50mM)	1.5ul	
iv.	gDNA(10ng/ul)	10ul	* or 0 for the Control tubes
v.	H_2O	6.75	* or 16.75 for the Control tubes
vi.	Primer 1 (25uM)	1ul	
vii.	Primer 2 (25uM)	1ul	* pipette mixture up and down 4-5 times
viii.	Taq enzyme (1U/ul)	1ul	
ix.	Final volume	25ul	

x. Briefly perform a Quick-spin to collect the reagents at the bottom of the tube

f. Although the T_ms of the primers indicate that an annealing temperature of ~57°C will be efficient, better specificity is usually obtained using a temperature somewhat above the T_m. Thus we will try two temperatures, using two thermocyclers. The following are the conditions:

i.	Melt		95°C	5 minutes
ii.	Cycling x 30			
		a.	94°C	30sec
		b.	57°C	30sec ** or 64°C
		c.	72°C	30sec
iii.	Finishing		72°C	7 minutes
iv.	Hold		4°C	indefinite

3. To test the effectiveness of the PCR reaction, you will run an acrylamide gel. Note that the Absorbance would include the unincorporated nucleotides so we don't take a spec reading.
   a. Pour an 8% polyacrylamide gel in 1X TBE, with 10 wells.
   b. Dilute 1ul of the PCR product with 19ul of 1X TE buffer.
   c. To 3ul of the diluted product add 1 ul of 4X loading dye.
   d. To 6 ul of the diluted product add 2 ul of 4X loading dye.
   e. You should have 8 products (including controls) to load on the gel.

   f. Use the Bionexus All Purpose Hi-Lo DNA markers, with 1ul per lane (this include a 400 bp band).
   g. Electrophorese at 100V for 3 hours.
   h. Stain in SYBR Green I dye (5 ul on 50 ml of water, wait 20 minutes, then image).
   i. Using the ladder and the mass of bands in the ladder estimate the size and yield of the propducts.
4. Add 4 ul of 3M NaOAc and 60ul of 95% ethanol to your PCR sample tubes. Mix well and store at -20C (we may use these for Sanger sequencing later in the semester).

Note: some of the plants are flowering – this is the second of the phenotypes we are following. Please check your plants and record (written or with a picture) the color of the blossoms.